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(54) Title: NEW USE

(57) Abstract: The use of 11CBY polypeptides and polynucleotides in the design of protocols for the treatment of stroke, pain, or neuropathies, among others, and diagnostic assays for such conditions. Also disclosed are methods for producing such polypeptides by recombinant techniques.

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(Exhibit 2)

New Use

Field of the Invention

This invention relates to new uses for polynucleotides and polypeptides encoded by them, to their use in therapy and in identifying compounds which may be agonists, antagonists and /or inhibitors which are potentially useful in therapy.

Summary of the Invention

In one aspect, the invention relates to new uses of 11CBY polynucleotides and polypeptides disclosed in WO96/18651 (SmithKline Beecham). Such uses include the treatment of stroke, pain and neuropathies hereinafter referred to as "the Diseases", amongst others In another aspect the invention relates to 11CBY recombinant materials and methods for their production In a further aspect, the invention relates to methods for identifying agonists and antagonists/inhibitors using the materials provided by the invention, and treating conditions associated with 11CBY imbalance or mutation with the identified compounds In a still further aspect, the invention relates to diagnostic assays for detecting diseases or disorders associated with inappropriate 11CBY activity or levels.

Description of the Invention

In a first aspect, the present invention relates to the use of a compound selected from:

(a) an 11CBY polypeptide;

(b) a compound which activates an 11CBY polypeptide; or

(c) a compound which inhibits an 11CBY polypeptide; or

(d) a polynucleotide encoding an 11CBY polypeptide,

for the manufacture of a medicament for treating:

(i) stroke;

(ii) pain; or

(iii) neuropathies.

Such 11CBY polypeptides include isolated polypeptides comprising an amino acid sequence which has at least 95% identity, preferably at least 97-99% identity, to that of SEQ ID NO:2 over the entire length of SEQ ID NO:2. Such polypeptides include those comprising the amino acid of SEQ ID NO:2.

Further polypeptides of the present invention include isolated polypeptides in which the amino acid sequence has at least 95% identity, preferably at least 97-99% identity, to the amino

acid sequence of SEQ ID NO:2 over the entire length of SEQ ID NO:2. Such polypeptides include the polypeptide of SEQ ID NO:2.

Further peptides of the present invention include isolated polypeptides encoded by a polynucleotide comprising the sequence contained in SEQ ID NO:1.

The 11CBY polypeptides of the present invention are members of the G-Protein coupled receptor superfamily. The polypeptides show the highest homology with somatostatin receptors, in terms of amino acid sequence identity, but the polypeptides have been shown not to be functional receptors for the somatostatin/corticostatin ligand family. Instead, the polypeptides of the present invention have been shown to bind the melanin-concentrating hormone (MCH) (Chambers et al. (1999) *Nature*, 400, 261-265; Saito et al. (1999) *Nature*, 400, 265-269; Bachner D et al. (1999) *FEBS Lett.*, 457(3), 522-524; Lembo PMC et al. (1999) *Nature Cell Biol.*, 1, 267-271; Shimonura, T et al. (1999) *Biochem. Biophys. Res. Commun.*, 261(3), 622-626). Thus the 11CBY polypeptides of the invention are believed to be the physiological MCH receptor. The rat ortholog of the human 11CBY receptor is called SLC-1 (Lakaye et al. (1998) Biochimica et Biophysica Acta 1401, 216-220).

The MCH receptor is believed to be important for the regulation of brain functions and body homeostasis. Its preferential localisation within brain nuclei, suggesting a major role in food intake (Chambers et al. 1999), means that this receptor could be the receptor mediating the orexigenic properties of the ligand MCH (Qu et al., (1996) *Nature*, 380, 243-247). In support of this, it has been shown that "knock-out" mice (deleted for the mouse MCH receptor) are lean and hypophagic (Shimada, M et al., (1998) *Nature*, 396, 670-674). Thus, drugs targeting the MCH receptor could be useful in the treatment of feeding disorders, eg. obesity and anorexia.

Also it is believed that drugs acting on the MCH receptor could be useful in the treatment of depression given the involvement of MCH in the hypothalamo-pituitary-adrenal regulation (see Nahon (1994) *Crit. Rev. in Neurobiol.*, 8(4), 221-262) and the presence of the MCH receptor in key brain areas dealing with the regulation of the stress response (such as the ventricular nuclei of the hypothalamus, the amygdaloid regions, the hippocampal formation; see Kolakowski et al. (1996); Chambers et al, (1999)).

These properties are hereinafter referred to as " 11CBY activity" or " 11CBY polypeptide activity" or "biological activity of 11CBY ". Also included amongst these activities are antigenic and immunogenic activities of said 11CBY polypeptides, in particular the antigenic and immunogenic activities of the polypeptide of SEQ ID NO:2. Preferably, a polypeptide of the present invention exhibits at least one biological activity of 11CBY .

The polypeptides of the present invention may be in the form of the "mature" protein or may be a part of a larger protein such as a precursor or a fusion protein. It is often advantageous to include an additional amino acid sequence which contains secretory or leader sequences, pro-sequences, sequences which aid in purification such as multiple histidine residues, or an additional sequence for stability during recombinant production.

The present invention also includes variants of the aforementioned polypeptides, that is polypeptides that vary from the referents by conservative amino acid substitutions, whereby a residue is substituted by another with like characteristics. Typical such substitutions are among Ala, Val, Leu and Ile; among Ser and Thr; among the acidic residues Asp and Glu; among Asn and Gln; and among the basic residues Lys and Arg; or aromatic residues Phe and Tyr. Particularly preferred are variants in which several, 5-10, 1-5, 1-3, 1-2 or 1 amino acids are substituted, deleted, or added in any combination.

Polypeptides of the present invention can be prepared in any suitable manner. Such polypeptides include isolated naturally occurring polypeptides, recombinantly produced polypeptides, synthetically produced polypeptides, or polypeptides produced by a combination of these methods. Means for preparing such polypeptides are well understood in the art.

In a further aspect, the present invention relates to 11CBY polynucleotides. Such polynucleotides include isolated polynucleotides comprising a nucleotide sequence encoding a polypeptide which has at least 95% identity to the amino acid sequence of SEQ ID NO:2, over the entire length of SEQ ID NO:2. In this regard, polypeptides which have at least 97% identity are highly preferred, whilst those with at least 98-99% identity are more highly preferred, and those with at least 99% identity are most highly preferred. Such polynucleotides include a polynucleotide comprising the nucleotide sequence contained in SEQ ID NO:1 encoding the polypeptide of SEQ ID NO:2.

Further polynucleotides of the present invention include isolated polynucleotides comprising a nucleotide sequence which has at least 95% identity to SEQ ID NO:1 over the entire length of SEQ ID NO:1. In this regard, polynucleotides which have at least 97% identity are highly preferred, whilst those with at least 98-99% identiy are more highly preferred, and those with at least 99% identity are most highly preferred. Such polynucleotides include a polynucleotide comprising the polynucleotide of SEQ ID NO:1 as well as the polynucleotide of SEQ ID NO:1.

The invention also provides polynucleotides which are complementary to all the above described polynucleotides.

The nucleotide sequence of SEQ ID NO:1 is a human cDNA sequence and comprises a polypeptide encoding sequence (243 to 1301) encoding a polypeptide of 353 amino acids, the

polypeptide of SEQ ID NO:2. The nucleotide sequence encoding the polypeptide of SEQ ID NO:2 may be identical to the polypeptide encoding sequence contained in SEQ ID NO:1 or it may be a sequence other than the one contained in SEQ ID NO:1, which, as a result of the redundancy (degeneracy) of the genetic code, also encodes the polypeptide of SEQ ID NO:2.

Preferred polypeptides and polynucleotides of the present invention are expected to have, *inter alia*, similar biological functions/properties to their homologous polypeptides and polynucleotides. Furthermore, preferred polypeptides and polynucleotides of the present invention have at least one 11CBY activity.

Polynucleotides of the present invention may be obtained, using standard cloning and screening techniques (Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.(1989)), from a cDNA library derived from mRNA in cells of human foetal brain, adrenal gland or ovary tumour. Polynucleotides of the invention can also be obtained from natural sources such as genomic DNA libraries or can be synthesized using well known and commercially available techniques.

When polynucleotides of the present invention are used for the recombinant production of polypeptides of the present invention, the polynucleotide may include the coding sequence for the mature polypeptide, by itself; or the coding sequence for the mature polypeptide in reading frame with other coding sequences, such as those encoding a leader or secretory sequence, a pre-, or pro- or prepro- protein sequence, or other fusion peptide portions. For example, a marker sequence which facilitates purification of the fused polypeptide can be encoded. In certain preferred embodiments of this aspect of the invention, the marker sequence is a hexa-histidine peptide, as provided in the pQE vector (Qiagen, Inc.) and described in Gentz *et al.*, Proc Natl Acad Sci USA (1989) 86:821-824, or is an HA tag. The polynucleotide may also contain non-coding 5' and 3' sequences, such as transcribed, non-translated sequences, splicing and polyadenylation signals, ribosome binding sites and sequences that stabilize mRNA.

Further embodiments of the present invention include polynucleotides encoding polypeptide variants which comprise the amino acid sequence of SEQ ID NO:2 and in which several, for instance from 5 to 10, 1 to 5, 1 to 3, 1 to 2 or 1, amino acid residues are substituted, deleted or added, in any combination.

Recombinant polypeptides of the present invention may be prepared by processes well known in the art from genetically engineered host cells comprising expression systems. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention.

For recombinant production, host cells can be genetically engineered to incorporate expression systems or portions thereof for polynucleotides of the present invention. Introduction of polynucleotides into host cells can be effected by methods described in many standard laboratory manuals, such as Davis et al., Basic Methods in Molecular Biology (1986) and Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989). Preferred such methods include, for instance, calcium phosphate transfection, DEAE-dextran mediated transfection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, scrape loading, ballistic introduction or infection.

Representative examples of appropriate hosts include bacterial cells, such as *Streptococci*, *Staphylococci*, *E. coli*, *Streptomyces* and *Bacillus subtilis* cells; fungal cells, such as yeast cells and *Aspergillus* cells; insect cells such as *Drosophila* S2 and *Spodoptera* Sf9 cells; animal cells such as CHO, COS, HeLa, C127, 3T3, BHK, HEK 293 and Bowes melanoma cells; and plant cells.

A great variety of expression systems can be used, for instance, chromosomal, episomal and virus-derived systems, e.g., vectors derived from bacterial plasmids, from bacteriophage, from transposons, from yeast episomes, from insertion elements, from yeast chromosomal elements, from viruses such as baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids. The expression systems may contain control regions that regulate as well as engender expression. Generally, any system or vector which is able to maintain, propagate or express a polynucleotide to produce a polypeptide in a host may be used. The appropriate nucleotide sequence may be inserted into an expression system by any of a variety of well-known and routine techniques, such as, for example, those set forth in Sambrook *et al.*, Molecular Cloning, A Laboratory Manual (supra). Appropriate secretion signals may be incorporated into the desired polypeptide to allow secretion of the translated protein into the lumen of the endoplasmic reticulum, the periplasmic space or the extracellular environment. These signals may be endogenous to the polypeptide or they may be heterologous signals.

Polypeptides of the present invention can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography is employed for purification. Well known techniques for refolding proteins may

be employed to regenerate active conformation when the polypeptide is denatured during isolation and or purification.

The polypeptides of the invention or their fragments or analogs thereof, or cells expressing them, can also be used as immunogens to produce antibodies immunospecific for polypeptides of the present invention. The term "immunospecific" means that the antibodies have substantially greater affinity for the polypeptides of the invention than their affinity for other related polypeptides in the prior art.

Antibodies generated against polypeptides of the present invention may be obtained by administering the polypeptides or epitope-bearing fragments, analogs or cells to an animal, preferably a non-human animal, using routine protocols. For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler, G. and Milstein, C., Nature (1975) 256:495-497), the trioma technique, the human B-cell hybridoma technique (Kozbor *et al.*, Immunology Today (1983) 4:72) and the EBV-hybridoma technique (Cole *et al.*, Monoclonal Antibodies and Cancer Therapy, 77-96, Alan R. Liss, Inc., 1985).

Techniques for the production of single chain antibodies, such as those described in U.S. Patent No. 4,946,778, can also be adapted to produce single chain antibodies to polypeptides of this invention. Also, transgenic mice, or other organisms, including other mammals, may be used to express humanized antibodies.

The above-described antibodies may be employed to isolate or to identify clones expressing the polypeptide or to purify the polypeptides by affinity chromatography.

Antibodies against polypeptides of the present invention may also be employed to treat the Diseases, amongst others.

In a further aspect, the present invention relates to genetically engineered soluble fusion proteins comprising a polypeptide of the present invention, or a fragment thereof, and various portions of the constant regions of heavy or light chains of immunoglobulins of various subclasses (IgG, IgM, IgA, IgE). Preferred as an immunoglobulin is the constant part of the heavy chain of human IgG, particularly IgG1, where fusion takes place at the hinge region. In a particular embodiment, the Fc part can be removed simply by incorporation of a cleavage sequence which can be cleaved with blood clotting factor Xa. Furthermore, this invention relates to processes for the preparation of these fusion proteins by genetic engineering, and to the use thereof for drug screening, diagnosis and therapy. A further aspect of the invention also relates to polynucleotides encoding such fusion proteins. Examples of fusion protein technology can be found in International Patent Application Nos. WO94/29458 and WO94/22914.

Another aspect of the invention relates to a method for inducing an immunological response in a mammal which comprises inoculating the mammal with a polypeptide of the present invention, adequate to produce antibody and/or T cell immune response to protect said animal from the Diseases hereinbefore mentioned, amongst others. Yet another aspect of the invention relates to a method of inducing immunological response in a mammal which comprises, delivering a polypeptide of the present invention *via* a vector directing expression of the polynucleotide and coding for the polypeptide *in vivo* in order to induce such an immunological response to produce antibody to protect said animal from diseases.

A further aspect of the invention relates to an immunological/vaccine formulation (composition) which, when introduced into a mammalian host, induces an immunological response in that mammal to a polypeptide of the present invention wherein the composition comprises a polypeptide or polynucleotide of the present invention The vaccine formulation may further comprise a suitable carrier. Since a polypeptide may be broken down in the stomach, it is preferably administered parenterally (for instance, subcutaneous, intramuscular, intravenous, or intradermal injection). Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation instonic with the blood of the recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents or thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampoules and vials and may be stored in a freeze-dried condition requiring only the addition of the sterile liquid carrier immediately prior to use. The vaccine formulation may also include adjuvant systems for enhancing the immunogenicity of the formulation, such as oil-in water systems and other systems known in the art. The dosage will depend on the specific activity of the vaccine and can be readily determined by routine experimentation.

11CBY polypeptides are responsible for one or more biological functions, including one or more disease states, in particular the Diseases hereinbefore mentioned. It is therefore desirous to devise screening methods to identify compounds which stimulate or which inhibit the function of the polypeptide. Accordingly, in a further aspect, the present invention provides for a method of screening compounds to identify those which stimulate or which inhibit the function of the polypeptide. In general, agonists or antagonists may be employed for therapeutic and prophylactic purposes for such Diseases as hereinbefore mentioned. Compounds may be identified from a variety of sources, for example, cells, cell-free preparations, chemical libraries, and natural product mixtures. Such agonists, antagonists or inhibitors so-identified may be natural or modified substrates, ligands, receptors, enzymes, etc., as the case may be, of the polypeptide; or may be

structural or functional mimetics thereof (see Coligan *et al.*, Current Protocols in Immunology 1(2):Chapter 5 (1991)).

The screening method may simply measure the binding of a candidate compound to the polypeptide, or to cells or membranes bearing the polypeptide, or a fusion protein thereof by means of a label directly or indirectly associated with the candidate compound. Alternatively, the screening method may involve competition with a labeled competitor, for example labelled MCH. Further, these screening methods may test whether the candidate compound results in a signal generated by activation or inhibition of the polypeptide, using detection systems appropriate to the cells bearing the polypeptide. Inhibitors of activation are generally assayed in the presence of a known agonist and the effect on activation by the agonist by the presence of the candidate compound is observed. Constitutively active polpypeptides may be employed in screening methods for inverse agonists or inhibitors, in the absence of an agonist or inhibitor, by testing whether the candidate compound results in inhibition of activation of the polypeptide. Further, the screening methods may simply comprise the steps of mixing a candidate compound with a solution containing a polypeptide of the present invention, to form a mixture, measuring 11CBY activity in the mixture, and comparing the 11CBY activity of the mixture to a standard. Fusion proteins, such as those made from Fc portion and 11CBY polypeptide, as hereinbefore described, can also be used for high-throughput screening assays to identify antagonists for the polypeptide of the present invention (see D. Bennett *et al.*, J Mol Recognition, 8:52-58 (1995); and K. Johanson *et al.*, J Biol Chem, 270(16):9459-9471 (1995)).

The polynucleotides, polypeptides and antibodies to the polypeptide of the present invention may also be used to configure screening methods for detecting the effect of added compounds on the production of mRNA and polypeptide in cells. For example, an ELISA assay may be constructed for measuring secreted or cell associated levels of polypeptide using monoclonal and polyclonal antibodies by standard methods known in the art. This can be used to discover agents which may inhibit or enhance the production of polypeptide (also called antagonist or agonist, respectively) from suitably manipulated cells or tissues.

The polypeptide may be used to identify interacting proteins or other molecules. For example the identification of interacting kinases may help to elucidate the signalling pathway of which 11CBY forms a part. These methods include, but are not limited to, two-hybrid system (Fields and Song, Nature 340, pp. 245-246 (1989); Durfee et al., Genes Dev. 7, pp. 555-569 (1993); Bartel and Fields, Methods in Enzymology 254, pp. 241-262 (1995)), λgt11 expression cloning (Blackwood and Eisenmann, Methods in Enzymology 254, pp. 229-240 (1995)), expression screening for protein kinase substrates (Fukunaga and Hunter, EMBO J. 16, pp. 1921-

1933 (1997)) as well as coimmunoprecipitation and western blotting assays (Ransone, Methods in Enzymology 254, pp. 491-496 (1995), Okamura et al., Methods in Enzymology 254, pp. 535-549 (1995)). These screening methods may also be used to identify agonists and antagonists of the polypeptide which compete with the binding of the polypeptide to its receptors, if any. Standard methods for conducting such assays are well understood in the art.

Examples of potential polypeptide antagonists include antibodies or, in some cases, oligonucleotides or proteins which are closely related to the ligands, receptors etc., of the polypeptide, e.g., a fragment of the ligands, receptors etc.; or small molecules which bind to the polypetide of the present invention but do not elicit a response, so that the activity of the polypeptide is prevented.

Thus, in another aspect, the present invention relates to a screening kit for identifying agonists, antagonists, ligands or receptors etc. for polypeptides of the present invention; or compounds which decrease or enhance the production of such polypeptides, which comprises:
(a) a polypeptide of the present invention;
(b) a recombinant cell expressing a polypeptide of the present invention;
(c) a cell membrane expressing a polypeptide of the present invention; or
(d) antibody to a polypeptide of the present invention;
which polypeptide is preferably that of SEQ ID NO:2.

It will be appreciated that in any such kit, (a), (b), (c) or (d) may comprise a substantial component.

It will be readily appreciated by the skilled artisan that a polypeptide of the present invention may also be used in a method for the structure-based design of an agonist, antagonist or inhibitor of the polypeptide, by:
(a) determining in the first instance the three-dimensional structure of the polypeptide;
(b) deducing the three-dimensional structure for the likely reactive or binding site(s) of an agonist, antagonist or inhibitor;
(c) synthesing candidate compounds that are predicted to bind to or react with the deduced binding or reactive site; and
(d) testing whether the candidate compounds are indeed agonists, antagonists or inhibitors.
It will be further appreciated that this will normally be an interative process.

In a further aspect, the present invention provides methods of treating abnormal conditions such as, for instance, stroke, pain or neuropathies, related to either an excess of, or an under-expression of, 11CBY polypeptide activity.

If the activity of the polypeptide is in excess, several approaches are available. One approach comprises administering to a subject in need thereof an inhibitor compound (antagonist) as hereinabove described, optionally in combination with a pharmaceutically acceptable carrier, in an amount effective to inhibit the function of the polypeptide, such as, for example, by blocking the binding of ligands, substrates, receptors, enzymes, etc., or by inhibiting a second signal, and thereby alleviating the abnormal condition. In another approach, soluble forms of the polypeptides still capable of binding the ligand, substrate, enzymes, receptors, etc. in competition with endogenous polypeptide may be administered. Typical examples of such competitors include fragments of the 11CBY polypeptide.

In still another approach, expression of the gene encoding endogenous 11CBY polypeptide can be inhibited using expression blocking techniques. Known such techniques involve the use of antisense sequences, either internally generated or externally administered (see, for example, O'Connor, J Neurochem (1991) 56:560 in Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988)). Alternatively, oligonucleotides which form triple helices ("triplexes") with the gene can be supplied (see, for example, Lee *et al.*, Nucleic Acids Res (1979) 6:3073; Cooney *et al.*, Science (1988) 241:456; Dervan *et al.*, Science (1991) 251:1360). These oligomers can be administered *per se* or the relevant oligomers can be expressed *in vivo*. Synthetic antisense or triplex oligonucleotides may comprise modified bases or modified backbones. Examples of the latter include methylphosphonate, phosphorothioate or peptide nucleic acid backbones. Such backbones are incorporated in the antisense or triplex oligonucleotide in order to provide protection from degradation by nucleases and are well known in the art. Antisense and triplex molecules synthesised with these or other modified backbones also form part of the present invention.

In addition, expression of the human 11CBY polypeptide may be prevented by using ribozymes specific to the human 11CBY mRNA sequence. Ribozymes are catalytically active RNAs that can be natural or synthetic (see for example Usman, N, et al., Curr. Opin. Struct. Biol (1996) 6(4), 527-33.) Synthetic ribozymes can be designed to specifically cleave human 11CBY mRNAs at selected positions thereby preventing translation of the human 11CBY mRNAs into functional polypeptide. Ribozymes may be synthesised with a natural ribose phosphate backbone and natural bases, as normally found in RNA molecules. Alternatively the ribozymes may be synthesised with non-natural backbones to provide protection from ribonuclease degradation, for example, 2'-O-methyl RNA, and may contain modified bases.

For treating abnormal conditions related to an under-expression of 11CBY and its activity, several approaches are also available. One approach comprises administering to a subject a

therapeutically effective amount of a compound which activatesa polypeptide of the present invention, i.e., an agonist as described above, in combination with a pharmaceutically acceptable carrier, to thereby alleviate the abnormal condition. Alternatively, gene therapy may be employed to effect the endogenous production of 11CBY by the relevant cells in the subject. For example, a polynucleotide of the invention may be engineered for expression in a replication defective retroviral vector, as discussed above. The retroviral expression construct may then be isolated and introduced into a packaging cell transduced with a retroviral plasmid vector containing RNA encoding a polypeptide of the present invention such that the packaging cell now produces infectious viral particles containing the gene of interest. These producer cells may be administered to a subject for engineering cells *in vivo* and expression of the polypeptide *in vivo*. For an overview of gene therapy, see Chapter 20, Gene Therapy and other Molecular Genetic-based Therapeutic Approaches, (and references cited therein) in Human Molecular Genetics, T Strachan and A P Read, BIOS Scientific Publishers Ltd (1996). Another approach is to administer a therapeutic amount of a polypeptide of the present invention in combination with a suitable pharmaceutical carrier.

In a further aspect, the present invention provides for pharmaceutical compositions comprising a therapeutically effective amount of a polypeptide, such as the soluble form of a polypeptide of the present invention, agonist/antagonist peptide or small molecule compound, in combination with a pharmaceutically acceptable carrier or excipient. Such carriers include, but are not limited to, saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The invention further relates to pharmaceutical packs and kits comprising one or more containers filled with one or more of the ingredients of the aforementioned compositions of the invention. Polypeptides and other compounds of the present invention may be employed alone or in conjunction with other compounds, such as therapeutic compounds.

The composition will be adapted to the route of administration, for instance by a systemic or an oral route. Preferred forms of systemic administration include injection, typically by intravenous injection. Other injection routes, such as subcutaneous, intramuscular, or intraperitoneal, can be used. Alternative means for systemic administration include transmucosal and transdermal administration using penetrants such as bile salts or fusidic acids or other detergents. In addition, if a polypeptide or other compounds of the present invention can be formulated in an enteric or an encapsulated formulation, oral administration may also be possible. Administration of these compounds may also be topical and/or localized, in the form of salves, pastes, gels, and the like.

The dosage range required depends on the choice of peptide or other compounds of the present invention, the route of administration, the nature of the formulation, the nature of the subject's condition, and the judgment of the attending practitioner. Suitable dosages, however, are

in the range of 0.1-100 μg/kg of subject. Wide variations in the needed dosage, however, are to be expected in view of the variety of compounds available and the differing efficiencies of various routes of administration. For example, oral administration would be expected to require higher dosages than administration by intravenous injection. Variations in these dosage levels can be adjusted using standard empirical routines for optimization, as is well understood in the art.

Polypeptides used in treatment can also be generated endogenously in the subject, in treatment modalities often referred to as "gene therapy" as described above. Thus, for example, cells from a subject may be engineered with a polynucleotide, such as a DNA or RNA, to encode a polypeptide *ex vivo*, and for example, by the use of a retroviral plasmid vector. The cells are then introduced into the subject.

The following definitions are provided to facilitate understanding of certain terms used frequently hereinbefore.

"Antibodies" as used herein includes polyclonal and monoclonal antibodies, chimeric, single chain, and humanized antibodies, as well as Fab fragments, including the products of an Fab or other immunoglobulin expression library.

"Isolated" means altered "by the hand of man" from the natural state. If an "isolated" composition or substance occurs in nature, it has been changed or removed from its original environment, or both. For example, a polynucleotide or a polypeptide naturally present in a living animal is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein.

"Polynucleotide" generally refers to any polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. "Polynucleotides" include, without limitation, single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, "polynucleotide" refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The term "polynucleotide" also includes DNAs or RNAs containing one or more modified bases and DNAs or RNAs with backbones modified for stability or for other reasons. "Modified" bases include, for example, tritylated bases and unusual bases such as inosine. A variety of modifications may be made to DNA and RNA; thus, "polynucleotide" embraces chemically, enzymatically or metabolically modified forms of polynucleotides as typically found in nature, as well as the chemical forms of DNA and RNA characteristic of viruses and cells.

"Polynucleotide" also embraces relatively short polynucleotides, often referred to as oligonucleotides.

"Polypeptide" refers to any peptide or protein comprising two or more amino acids joined to each other by peptide bonds or modified peptide bonds, i.e., peptide isosteres. "Polypeptide" refers to both short chains, commonly referred to as peptides, oligopeptides or oligomers, and to longer chains, generally referred to as proteins. Polypeptides may contain amino acids other than the 20 gene-encoded amino acids. "Polypeptides" include amino acid sequences modified either by natural processes, such as post-translational processing, or by chemical modification techniques which are well known in the art. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature. Modifications may occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present to the same or varying degrees at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications. Polypeptides may be branched as a result of ubiquitination, and they may be cyclic, with or without branching. Cyclic, branched and branched cyclic polypeptides may result from post-translation natural processes or may be made by synthetic methods. Modifications include acetylation, acylation, ADP-ribosylation, amidation, biotinylation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cystine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination (see, for instance, Proteins - Structure and Molecular Properties, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York, 1993; Wold, F., Post-translational Protein Modifications: Perspectives and Prospects, pgs. 1-12 in Post-translational Covalent Modification of Proteins, B. C. Johnson, Ed., Academic Press, New York, 1983; Seifter *et al.*, "Analysis for protein modifications and nonprotein cofactors", Meth Enzymol (1990) 182:626-646 and Rattan *et al.*, "Protein Synthesis: Post-translational Modifications and Aging", Ann NY Acad Sci (1992) 663:48-62).

"Variant" refers to a polynucleotide or polypeptide that differs from a reference polynucleotide or polypeptide, but retains essential properties. A typical variant of a polynucleotide differs in nucleotide sequence from another, reference polynucleotide. Changes

in the nucleotide sequence of the variant may or may not alter the amino acid sequence of a polypeptide encoded by the reference polynucleotide. Nucleotide changes may result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by the reference sequence, as discussed below. A typical variant of a polypeptide differs in amino acid sequence from another, reference polypeptide. Generally, differences are limited so that the sequences of the reference polypeptide and the variant are closely similar overall and, in many regions, identical. A variant and reference polypeptide may differ in amino acid sequence by one or more substitutions, additions, deletions in any combination. A substituted or inserted amino acid residue may or may not be one encoded by the genetic code. A variant of a polynucleotide or polypeptide may be a naturally occurring such as an allelic variant, or it may be a variant that is not known to occur naturally. Non-naturally occurring variants of polynucleotides and polypeptides may be made by mutagenesis techniques or by direct synthesis.

"Identity," as known in the art, is a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness between polypeptide or polynucleotide sequences, as the case may be, as determined by the match between strings of such sequences. "Identity" and "similarity" can be readily calculated by known methods, including but not limited to those described in (*Computational Molecular Biology*, Lesk, A.M., ed., Oxford University Press, New York, 1988; *Biocomputing: Informatics and Genome Projects*, Smith, D.W., ed., Academic Press, New York, 1993; *Computer Analysis of Sequence Data*, Part I, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; *Sequence Analysis in Molecular Biology*, von Heinje, G., Academic Press, 1987; and *Sequence Analysis Primer*, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; and Carillo, H., and Lipman, D., SIAM *J. Applied Math., 48:* 1073 (1988). Preferred methods to determine identity are designed to give the largest match between the sequences tested. Methods to determine identity and similarity are codified in publicly available computer programs. Preferred computer program methods to determine identity and similarity between two sequences include, but are not limited to, the GCG program package (Devereux, J., et al., *Nucleic Acids Research 12(1):* 387 (1984)), BLASTP, BLASTN, and FASTA (Atschul, S.F. et al., *J. Molec. Biol. 215:* 403-410 (1990). The BLAST X program is publicly available from NCBI and other sources (*BLAST Manual*, Altschul, S., *et al.*, NCBI NLM NIH Bethesda, MD 20894; Altschul, S., *et al.*, *J. Mol. Biol. 215*: 403-410 (1990). The well known Smith Waterman algorithm may also be used to determine identity.

Preferred parameters for polypeptide sequence comparison include the following:

1) Algorithm: Needleman and Wunsch, J. Mol Biol. 48: 443-453 (1970)

Comparison matrix: BLOSSUM62 from Hentikoff and Hentikoff, Proc. Natl. Acad. Sci. USA. 89:10915-10919 (1992)

Gap Penalty: 12

Gap Length Penalty: 4

A program useful with these parameters is publicly available as the "gap" program from Genetics Computer Group, Madison WI. The aforementioned parameters are the default parameters for peptide comparisons (along with no penalty for end gaps).

Preferred parameters for polynucleotide comparison include the following:

1) Algorithm: Needleman and Wunsch, J. Mol Biol. 48: 443-453 (1970)

Comparison matrix: matches = +10, mismatch = 0

Gap Penalty: 50

Gap Length Penalty: 3

Available as: The "gap" program from Genetics Computer Group, Madison WI. These are the default parameters for nucleic acid comparisons.

By way of example, a polynucleotide sequence of the present invention may be identical to the reference sequence of SEQ ID NO:1, that is be 100% identical, or it may include up to a certain integer number of nucleotide alterations as compared to the reference sequence. Such alterations are selected from the group consisting of at least one nucleotide deletion, substitution, including transition and transversion, or insertion, and wherein said alterations may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among the nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence. The number of nucleotide alterations is determined by multiplying the total number of nucleotides in SEQ ID NO:1 by the numerical percent of the respective percent identity(divided by 100) and subtracting that product from said total number of nucleotides in SEQ ID NO:1, or:

$$n_n \leq x_n - (x_n \bullet y),$$

wherein n_n is the number of nucleotide alterations, x_n is the total number of nucleotides in SEQ ID NO:1, and y is, for instance, 0.70 for 70%, 0.80 for 80%, 0.85 for 85%, 0.90 for 90%, 0.95 for 95%,etc., and wherein any non-integer product of x_n and y is rounded down to the nearest integer prior to subtracting it from x_n. Alterations of a polynucleotide sequence encoding the polypeptide of SEQ ID NO:2 may create nonsense, missense or frameshift mutations in this

coding sequence and thereby alter the polypeptide encoded by the polynucleotide following such alterations.

Similarly, a polypeptide sequence of the present invention may be identical to the reference sequence of SEQ ID NO:2, that is be 100% identical, or it may include up to a certain integer number of amino acid alterations as compared to the reference sequence such that the % identity is less than 100%. Such alterations are selected from the group consisting of at least one amino acid deletion, substitution, including conservative and non-conservative substitution, or insertion, and wherein said alterations may occur at the amino- or carboxy-terminal positions of the reference polypeptide sequence or anywhere between those terminal positions, interspersed either individually among the amino acids in the reference sequence or in one or more contiguous groups within the reference sequence. The number of amino acid alterations for a given % identity is determined by multiplying the total number of amino acids in SEQ ID NO:2 by the numerical percent of the respective percent identity(divided by 100) and then subtracting that product from said total number of amino acids in SEQ ID NO:2, or:

$$n_a \leq x_a - (x_a \circ y),$$

wherein $\mathbf{n_a}$ is the number of amino acid alterations, $\mathbf{x_a}$ is the total number of amino acids in SEQ ID NO:2, and y is, for instance 0.70 for 70%, 0.80 for 80%, 0.85 for 85% etc., and wherein any non-integer product of $\mathbf{x_a}$ and y is rounded down to the nearest integer prior to subtracting it from $\mathbf{x_a}$.

"Homolog" is a generic term used in the art to indicate a polynucleotide or polypeptide sequence possessing a high degree of sequence relatedness to a subject sequence. Such relatedness may be quantified by determining the degree of identity and/or similarity between the sequences being compared as hereinbefore described. Falling within this generic term are the terms "ortholog", meaning a polynucleotide or polypeptide that is the functional equivalent of a polynucleotide or polypeptide in another species, and "paralog" meaning a functionally similar sequence when considered within the same species.

"Fusion protein" refers to a protein encoded by two, often unrelated, fused genes or fragments thereof. In one example, EP-A-0 464 discloses fusion proteins comprising various portions of constant region of immunoglobulin molecules together with another human protein or part thereof. In many cases, employing an immunoglobulin Fc region as a part of a fusion protein is advantageous for use in therapy and diagnosis resulting in, for example, improved pharmacokinetic properties [see, e.g., EP-A 0232 262]. On the other hand, for some uses it

would be desirable to be able to delete the Fc part after the fusion protein has been expressed, detected and purified.

Examples

Example 1 - Quantitative RT-PCR and mRNA localisation of SLC-1 in rat brain (Taqman analysis)

The SLC-1 terminology for the rat ortholog of the human 11CBY has been used for clarity and consistency with the terminology used in the art.

For mRNA localisation studies male Sprague-Dawley rats (300-350g) were sacrificed by CO_2 asphyxia followed by cervical dislocation. Sixteen brain regions and spinal cord and dorsal root ganglia were dissected free. Each tissue sample was pooled from between six and sixteen rats depending on the size of the individual tissue samples obtained. Total RNA was extracted from the tissue according to the manufacturer's suggested protocol with the addition of an extra chloroform extraction step and phase separation, and an extra wash of the isolated RNA in 70% ethanol. The RNA was resuspended in autoclaved, ultrapure water and the concentration calculated by A_{260} measurement. RNA quality was assessed by electrophoresis on a 1% agarose gel. First strand cDNA synthesis was carried out by oligo(dT) priming from 1μg of each RNA sample (0.01M DTT, 0.5mM each dNTP, 0.5μg oligo(dT) primer, 40U RNAseOUT ribonuclease inhibitor (Life Technologies) 200U Superscript II reverse transcriptase (Life Technologies)). Triplicate reverse transcription reactions were performed along with an additional reaction in which the reverse transcriptase was omitted to allow for assessment of genomic DNA contamination of the RNA. The resulting cDNA products were divided into twenty aliquots for parallel Taqman PCR reactions using different primer and probe sets for quantification of multiple cDNA sequences.

Quantitative RT-PCR was carried out using an ABI 7700 sequence detector (Perkin Elmer) on the cDNA samples (2.5mM $MgCl_2$, 0.2mM dATP, dCTP, dGTP and dUTP, 0.1μM each primer, 0.05M Taqman probe, 0.01U AmpErase uracil-N-glycosylase (Perkin Elmer), 0.0125U Amplitaq Gold DNA polymerase (Perkin Elmer); 50°C for 2 minutes, 95°C for 10 minutes followed by forty cycles of 95°C for 15 seconds, 60°C for 1 minute). Additional reactions were performed on each 96 well plate using known dilutions of rat genomic DNA as a PCR template to allow construction of a standard curve relating threshold cycle to template copy number.

Taqman primer and probe sets for SLC-1 and for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed using Primer Express software (Perkin Elmer). Parallel Taqman PCRs were run on each sample using the GAPDH primers and probe to

control for RNA integrity. Primer and probe sequences used were (forward primer, reverse primer and Taqman probe):

SLC-1 forward: 5'-CTCTACGCCAGGCTCATTCC (SEQ ID NO:3);

SLC-1 reverse: 5'-ACAGAGTGAACCAGTAGAGGTCAGTGT (SEQ ID NO:4);

SLC-1 labelled probe: 5'-GGCGGATGCCACAGCCCA (SEQ ID NO:5);

GAPDH forward: 5'-GAACATCATCCCTGCATCCA (SEQ ID NO:6);

GAPDH reverse: 5'-CCAGTGAGCTTCCCGTTCA (SEQ ID NO:7);

GAPDH labelled probe: 5'-CTTGCCCACAGCCTTGGCAGC (SEQ ID NO:8).

Copy numbers obtained for SLC-1 were normalised to those for GAPDH and the resulting values expressed as arbitrary units.

The primary data generated by Taqman RT-PCR consists of a threshold cycle value, indicating the PCR cycle at which the PCR product is detectable above an arbitrary threshold. The system was calibrated using known numbers of copies of genomic DNA. When the threshold cycle generated by these standards is plotted against the genomic DNA copy number on a logarithmic scale, the data points lie on a straight line. Using the threshold cycle values resulting from the cDNA samples from various tissues, a copy number can be read from this calibration curve.

In this experiment copy numbers for SLC-1 were normalised to GAPDH copies to control for variations in RNA quality and loading. Only slight variations in GAPDH mRNA expression were observed between the tissues that were included in the experiment. The results showed that SLC-1 is widely expressed in the rat nervous system, with mRNA detected in all tissues that were tested. However, some variation in expression is observed, with higher expression in amygdala, cerebral cortex (all divisions), hippocampus, hypothalamus and substantia nigra, and lower levels of expression in striatum, thalamus, cerebellum, rhombencephalon, spinal cord and dorsal root ganglia (DRG). The cerebral cortex and hippocampus are known to be important brain regions in the aetiology of schizophrenia.

Example 2 – In-situ hybridisation (ISH)

The ISH experiments were performed with oligonucleotides designed from the rat SLC-1 sequence (Lakaye et al. (1998) *supra*). Various oligonucleotides with non-overlapping sequences were designed using the PrimerSelect software of Lasergene. The program identified three sense sequences:

5'-GGCCACCGTCCACCCCATCTCCTCCAC -3' (SEQ ID NO:9);

5'-ATACTACAGCGCATGACGTCTTCGGTGGCCCC -3' (SEQ ID NO:10); and

5'- TCTGCAAACCTCGTTGCTGTCCACT-3' (SEQ ID NO:11).

(with their respective position on the rat SLC-1 cDNA sequence and length as follows: 429-455/27-mer; 691-722/32-mer; 6-30/25-mer). Sequences were checked for uniqueness using BLAST (Advanced search; GenEMBL complete).

Oligonucleotides were used independently and provided similar patterns of distribution. In order to enhance signal intensity, in some cases a 'cocktail' (equal molar mixture) of the oligoprobes was used. Full details of the ISH protocol have been reported elsewhere (Hervieu and Nahon (1995) Neuroendocrinology, 61, 348-364). Briefly, post-fixed rat brain sections (cut coronally or sagittally with a thickness of 20μm; post-fixed for 30 minutes in 4% PFA w/v in PBS) were hybridised overnight with [^{35}S] oligoprobes radiolabelled by 3'-tailing with terminal transferase (specific radiactivity > 10^9 dpm per microgram, incorporation > 80%), then were washed and dehydrated. The sections were exposed to Kodak Biomax film for up to 3 weeks. Control experiments included the use of the sense sequence oligoprobes, the use of excess (100x) cold antisense oligoprobes, and RnaseA-pretreated sections. Histological analysis was carried out as described in example 3.

In situ studies have shown dense localisation of rat SLC-1 mRNA in the rat septum, a region believed to be important in stroke. The in-situ studies also confirm the Taqman tissue localisation data discussed in example 1 concerning the expression of SLC-1 in the cerebral cortex and hippocampus.

Furthermore, the in-situ data shows a high level expression in the dorsal part of the spinal cord and in other regions dealing with nociception, for example peri-aqueductal gray matter, trigeminal nerve complex, ventral posterior nucleus of the thalamus and locus coeruleus, thus suggesting a role for rat SLC-1 and, by extrapolation, human 11CBY, in pain.

Example 3 – Immunochemistry

Antisera preparation and purification

Rabbit polyclonal antisera were raised against three different peptides derived from the human 11CBY sequence.

Name of the antiserum; Peptide sequence; Length	Position on the SLC-1 rat protein sequence	Localisation as compared to the predictive 7TM model	Sequence identity between the human and rat orthologs.
N: H-PTGPNASNISDGPDNLT-OH (17 amino-acid long) (SEQ ID NO:12)	9 to 25	N-terminal extracellular tail	88%; P residues replaced respectively by S and Q STGPNASNISDGQDNLT (SEQ ID NO:13)
M: H-SSTKFRKPSVA-OH (11 amino-acid long) (SEQ ID NO:14)	150 to 160	third intracellular loop	100%
C: H-SNAQTADEERTESKGT-OH (16 amino-acid long) (SEQ ID NO:15)	338 to 353	extreme C-terminal intracellular tail	100%

Table 1: human 11CBY sequences used to raise polyclonal antisera.

The peptides were synthesized using solid-phase methodology on a model 432A Applied Biosystem Synthethiser. Peptide purity was estimated by chromatography as being greater than 95%. The synthetic peptides were covalently NH2-coupled to the carrier Keyhole Limpet hemocyanin (KLH) using the glutaraldehyde method.

Two New Zealand rabbits were used for each peptide in the immunisation procedure. Initially rabbits were injected with 0.5 mg of the peptide-KLH conjugate in Freund's complete adjuvant, then subsequently boosted four times with the same amount of antigen suspended in incomplete adjuvant. The injections were repeated monthly. The bleeds were clotted overnight at 4°C and the serum separated from blood cells by centrifugation at 8, 000 x g. The sera were stored at -80°C until affinity purified.

Purification of the antisera

For the initial assessment of the antiserum specificity in immunohistochemical procedures, a micropurification procedure was performed using the immunogenic peptide linked

to BSA and bound to a 0.45μm nitrocellulose membrane (Sartorius, Gottingen, Germany). Crude antisera were incubated overnight at 4°C with 100μg of the peptide spotted on the filter in 100mM Tris-HCl pH=8.0, 0.05 % (v/v) Nonidet P-40, 150 mM NaCl and 0.5 % (w/v) Marvel fat-free dried milk. After three washes (10 min per wash in the same buffer), bound antibodies were eluted with 100mM glycine, pH2.5. The eluate was then neutralized with 1M Tris-HCl, pH8.0 (0.1 vol. of Tris per vol. of glycine).

Once the specificity of the antibody was checked (see below), a larger scale purification of the antisera was carried out using an affinity chromatography column. An N- terminal extended immunogenic peptide with a cysteine residue was coupled to the Sulfolink matrix following the manufacturer's recommendations (Pierce &Wariner, Chester, U.K). Three ml of the crude antiserum were applied to 1mg of the peptide covalently bound to 2ml of the solid gel in a 50ml tube on a rolling agitator overnight at 4°C. After the resin was washed with 100ml of 0.1 M PBS for 60 min at 4°C, specific antibodies were eluted with glycine in a similar procedure to that above, dialysed overnight at 4°C against 0.1M PBS and resuspended in storage buffer (0.1M PBS; 2 g/l BSA; 20 % (v/v) NGS; 30 % (v/v) glycerol). Routinely, the affinity-purified antibodies were eluted with 10ml of glycine and neutralized with 1ml of Tris-HCl, pH8. The purified antibodies were aliquoted and stored at -80°C until further use.

Immunohistochemistry (IHC)

Immunosignals were revealed with an ABC peroxidase reporter system as previously described (Hervieu and Emson (1998) Neuroscience,85, 4, 1263-1284) or by immunofluorescence. Briefly, adult male Sprague-Dawley rats were transcardiacally perfused with 4% paraformaldehyde in phosphate buffered saline (PBS). The brains were removed, post-fixed in 4% paraformaldehyde in (PBS) for 2 hours at 4°C and transferred into a 20% sucrose/PBS solution overnight. Following freezing of the brains in isopentane, 35μm coronal sections were cut in a cryostat. Sections were stored in 0.5% PFA in PBS at 4°C. Sections were incubated in PBS containing 20% methanol and 1.5% hydrogen peroxide for 30 minutes in order to quench the endogenous pseudo-peroxidase activity. Following a 2 x 5 minute washes in PBS, sections were placed in a blocking solution (3% normal goat serum, 2g/l bovine serum albumin, 0.1% TritonX 100 in PBS) for 30-45 minutes. Sections were then incubated with the primary antiserum for 48 hours at 4°C with gentle agitation. After primary incubation, sections were given three ten-minute washes in 0.3% TritonX 100 in PBS. Sections were then processed for peroxidase immunostaining using the Vector ABC (avidin:biotinylated-enzyme complex)

amplification system following the manufacturer recommendations. Sections were incubated for one hour at room temperature in Vector goat anti-rabbit IgG (H + L) with gentle shaking, then given three ten-minute washes in 0.3% TritonX 100 in PBS, and incubated under the same conditions in ABC in PBS (prepared at least 30 minutes prior to use). After a further three ten-minute washes in 0.3% TritonX 100 in PBS, sections were transferred into tris-buffered saline (TBS). Sections were incubated in 3,3'-diaminobenzidine (DAB) substrate (Vector Laboratories, U.K.) for 5-10 minutes before the reaction was stopped in distilled water. Sections were mounted onto Superfrost polished slides (BDH, U.K), allowed to airdry, then coverslipped with DPX mountant.

Immunochemical fluoresence was carried out as previously described (Hervieu and Nahon (1995) Neuroendocrinology, 61, 348-364) using one of several different streptavidin-conjugated fluorophores (Cy2, Cy3, Cy5, Red-Phycoerythrin, FITC, TRITC, AMCA; all from Jackson Laboratories, U.S.A.).

Immunocytochemistry (ICC)

Wild-type or transfected HEK 293 cells were grown on LabTek slides (Life Technologies, Paisley, U.K.) and fixed for 15 min in 4% w/v PFA in PBS 0.1 M. The fluorescence immunocytochemical procedure was similar to that already described for immunohistochemistry .

Immunochemical control procedures

Control experiments included the omission of the primary antiserum, the use of rabbit preimmune serum and pre-absorbing the antiserum with the immunogenic peptide. Pre-absorption controls were done with 10μM of the immunogenic peptide (incubated overnight with the antiserum prior to the incubation on sections). Specificity of the N, M and C antisera was investigated on SLC-1- HEK 293 transfected cells versus wild-type cells, with or without pre-absorption, with or without primary antisera in an ICCH fluorescence procedure as already reported (Chambers et al.,1999 *supra*). Specificity of the C antiserum has already been presented elsewhere (Chambers et al.,1999 *supra*).

Histological analysis:

Brain regions and nuclei were identified using the rat brain atlases of Paxinos and Watson (1998) The Rat Brain in Stereotaxic Coordinates, Third Edition, Academic Press, San Diego, for sagittal and horizontal sections and Swanson (1992) Brain maps: structure of the rat

brain, Elsevier, Amsterdam, for coronal sections. The nomenclature mainly follows that adopted in the report by Bittencourt et al. (1992) J. Comp. Neurol., 319, 218-245, describing the neuroanatomical distribution of the MCH system in the rat brain.

Image acquisition and processing

ISH data were captured with a Pulnix TM-765 black and white camera coupled to a TAMROM SP. Peroxidase IHC data were captured with a JVC 3CCD KY-F55B color video camera or a Leica DC 200 digital camera. Fluorescence IHC and ICC data were analysed with a Leica TCS confocal DM RB microscope. Confocal images were either taken as a 0.8µm thick single plane or as a stack of several planes (and thereafter reconstructed 3-D through a Projection function). Each single plane was averaged four times.

Image acquisition for ISH and peroxidase data was done with the Image ProPlus software (Media Cybernetics, U.S.A.). Images were taken with the highest level of contrast and brightness. Images were imported into PaintShop Pro Version 5.0 (Jasc Sofware, U.S.A.). Image modification involved only the transformation of color images into black and white images using a grey scale function, and adjusting the levels of brightness, contrast and red/green/blue balance, and some of the images had background filtered through a median cut noise function.

The results of these experiments showed, among other things, a high level expression in the dorsal part of the spinal cord and in other regions dealing with nociception (eg. peri-aqueductal gray matter, trigeminal nerve complex, ventral posterior nucleus of the thalamus, locus coeruleus), thus confirming the results of the in-situ hybridisation experiments discussed in example 2. Such localisation is consistent with a role for rat SLC-1 and therefore, by extrapolation, human 11CBY, in pain.

Example 4 – pMCAO (permanent middle cerebral artery occlusion) studies in rats

Suture Preparation

3/0 gauge monofilament nylon sutures were used for animals within the weight range 300-350 g. A 30 mm length of suture was heat- blunted at the tip to achieve a diameter of 0.26-0.30 mm. The diameter was checked using a micrometer under an operating microscope. The 20mm distal segment of the suture was then immersed in poly-L-lysine for five minutes (0.1% [wt/vol], in de-ionised water, 1:10 dilution of Sigma stock solution, as recommended) and left to dry overnight at room temperature. It has been established that suture diameter is not altered by the coating process (Stroke, 1995, 26 (12), 2313-2320).

Surgical Preparation

Male Sprague-Dawley rats (n=10 per group), supplied by Charles River, were used for the experiment. Rats were anaesthetised initially with a mixture of 4-5% halothane, 30% oxygen, and 70% nitrous oxide in an induction chamber, and maintained thereafter with a nitrous oxide/oxygen mixture containing 1.0-2.0% halothane delivered by a face mask. Body temperature was monitored throughout the surgical procedure by a rectal thermometer, and the animals maintained normothermic (37 ± 0.5^0C) via a heating blanket controlled by the thermometer. A needle temperature probe was also inserted into the left temporalis muscle to give an indirect measurement of brain temperature. Actual core and temporalis temperature values may be recorded at the time of MCA occlusion.

Induction of Focal Ischaemia

All animals underwent permanent MCA occlusion as described by Longa and co-workers (Stroke, 1989, 20, 84-91). The left carotid arteries were exposed through a midline cervical incision and sterile silk sutures (5/0) looped around the common carotid, external carotid, and internal carotid arteries. The arterial branches of the external carotid artery were all exposed and divided using diathermy forceps leaving a stump of ~2-3 mm in length, and a microvascular clip placed on the end of the stump to assist haemostasis. The pterygopalatine artery was exposed by developing a plane alongside the internal carotid artery, and ligated at its origin with fine silk (5/0) leaving the extracranial carotid circulation contiguous. Aneurysm clips were placed across the common carotid and internal carotid arteries and an arteriotomy was made in the external carotid artery stump allowing the introduction of a blunted, rounded length of monofilament nylon suture. This was secured in place with a silk suture and the aneurysm clip on the internal carotid artery removed. The suture was advanced into the internal carotid artery and passed into the intracranial circulation to lodge in the narrower lumen of the proximal anterior cerebral artery, approximately 17-21 mm distal to the carotid bifurcation, thereby occluding the origin of the MCA. The aneurysm clip on the common carotid artery was removed and haemostasis checked. The cervical wound was then sutured closed.

Post-Occlusion Recovery

Following MCA occlusion, anaesthesia was discontinued and the animals were allowed to regain consciousness and righting reflex under strict observation in an incubator (23-25^{O}C) for

1 hour. The animals were then housed individually in the post-operative recovery room, where their overall health status was closely monitored throughout the survival period.

Neurological Assessment

Motor and behavioural changes were assessed using a six-point grading scale at 1 hour following MCA occlusion, and prior to sacrifice up to 24 hours:

0, no deficit;
1, failure to extend right forepaw fully;
2, decreased grip of right forelimb while tail pulled;
3, spontaneous circling or walking to contralateral side;
4, walks only when stimulated with depressed level of consciousness;
5, unresponsive to stimulation.

Neuropathology and Quantification of Ischaemic Damage

Up to twenty-four hours after MCA occlusion (for lesion volume), rats were perfusion fixed with neutral buffered formalin (NBF) containing 5% sucrose. Rats were deeply anaesthetised with halothane in nitrous oxide/oxygen (70:30) and placed in a supine position. Anaesthesia was induced with 5% halothane, and maintained with 1-2%. The thorax was opened through a midline incision and an oral gavage needle inserted into the ascending aorta via the apex of the left ventricle and clamped in position. The right atrium was incised and 0.9% heparinised saline (10 IU/ml) infused at a pressure equal to the MABP of the animal (~100-120 mm Hg) until the effusate from the right atrium was bloodless (~ 50 ml). The animal was then perfused with NBF (~100-150 ml) at the same pressure. The animals were then decapitated and the brains stored in situ in NBF for 24 hrs, prior to dissection.

The brains were then serially sectioned (1.5mm intervals) through the cerebrum from the anterior poles to the cerebellum and the sections (50 um) stained with Cresyl Fast Violet. The sections were examined and the areas of ischaemic damage delineated at 8 pre-selected coronal levels from anterior +3.0mm to posterior -7.5mm relative to bregma. The areas of ischaemic damage at each coronal level were integrated with the known distance between each coronal level to derive the total volume of ischaemic damage in each animal. Volumes were then corrected for hemispheric swelling.

The volume of the cerebral hemispheres ipsilateral and contralateral to the occluded MCA were determined directly from the stained histological sections by integration from

assessment of total surface area at the same eight coronal planes employed in assessing areas of ischaemic damage. The difference between the two hemispheres provides a measure of the degree of associated brain swelling.

Expression of 11CBY and MCH mRNA in pMCAO rats

mRNA was extracted from rats submitted to pMCAO and levels were measured by Taqman (as described in example 1) in the cortex and the striatum. The upregulation of 11CBY mRNA as observed by Taqman in the pMCAO model (1day) is quite robust (4 times greater in the lesioned side of the cortex as compared to contralateral side and ~2.5 times greater in the in the lesioned side of the striatum as compared to contralateral side).

Levels of MCH mRNA were also elevated at 6 hours post-surgery and at 24 hours the MCH levels were 5 fold greater in the lesioned side of cortex and striatum than on the contralateral side.

These data support a therapeutic role in stroke for drugs targetting the 11CBY protein.

Example 5 - Expression of MCH and 11CBY mRNA in DRG and spinal cord in the rat Selzer model of neuropathic pain using Taqman quantitative PCR.

MCH and 11CBY mRNA levels were determined in tissues extracted from rats that had been prepared according to the neuropathic pain model developed by Selzer et al (1990) Pain 43: 205-218.

<u>Animals and tissue preparation</u>

Wistar rats (approx 200 g at the time of surgery, Harlan Olac, UK) were anaesthetized with isofluorine and a tight ligature (7.0 mersilk) placed around 30-50 % of the left sciatic nerve in the thigh. Sham animals were also prepared in which the sciatic nerve was exposed but not ligated. Animals were euthanased and the right (ipsilateral) and left (controlateral) L4-L5 DRG and the lumbar spinal cord extracted 6 hours, 1, 7, 14, 28 days after surgery. A total of 4 sham and 4 ligated animals were used for each experimental time-point. TaqMan PCR was used to evaluate the MCH and 11CBy mRNA levels in rat DRG and spinal cord at the above different time after partial nerve ligation of the sciatic nerve (Seltzer Z, et al (1990) Pain 43:205-218).

<u>RNA extraction and reverse transcription</u>

Total RNA was isolated (RNAzol B, Life Technology) and the RNA concentration calculated by A_{260} measurement. RNA quality was assessed by electrophoresis on a 1% agarose

gel. First strand cDNA was synthesised from 1 μg of each RNA sample; 0.01M DTT, 0.5 mM dNTP mix, 0.5 μg oligo(dT) primer, 40U RNaseOUT ribonuclease inhibitor (Life Technologies), 200U Superscript II reverse transcriptase (Life Technologies). An additional reaction was performed in which the reverse transcriptase enzyme was omitted in order to control for assessment of genomic DNA contamination in each sample.

Quantitative PCR

Taqman PCR was carried out using an ABI prism 7700 sequence detector (Perkin Elmer) on the cDNA samples. The PCR reaction (5 mM MgCl2, 0.3 mM dATP, dCTP, dGTP and 0.6 mM dUTP, 0.3 μM each primer, 0.2 μM Taqman probe, 0.01U AmpErase uracil-N-glycosylase, 1.25 U Amplitaq Gold DNA polymerase; all reagents from Perkin Elmer) was carried out at 50°C for 2 minutes, 95° C for 10 minutes followed by forty cycles of 95° C for 15 sec, 60° C for 1 minute.

In the present study the tissue levels of MCH and 11CBy mRNA were quantified by TaqMan using MCH and 11CBy mRNA primers and probes as used in example 1. TaqMan data were analysed using the C_T comparative method (Heid C. A., Stevens J., Livak K. J., Williams P. M. Real time quantitative PCR, Genome Res. 1996; 6: 968-994). The PCR efficiency of each target (MCH and 11CBy mRNA) and of the control (GAPDH) were equal over the cDNA concentrations used in these experiments. The amount of mRNA levels in the ligated tissues, normalized to the endogenous reference GAPDH and relative to the mRNA levels in the sham tissues was given by $(1+E\ \text{PCR efficiency})^{-\Delta\Delta CT}$ where $\Delta\Delta C_T = [C_T\ \text{Target} - C_T\ \text{GAPDH}]_{\text{Ligated}} - [C_T\ \text{Target} - C_T\ \text{GAPDH}]_{\text{Sham}}$ and C_T = PCR cycle at which the amplification plot crosses the the baseline threshold (Winer J. et al (1999) Anal. Biochem. 270(1):41-49).

It was found that MCH and the 11CBY gene were expressed in DRG and spinal cord from sham rats. Partial nerve ligation induced a significant increased in MCH and 11CBY mRNA levels in the left (ligated) DRG 7 days after sugery (4.59 fold and 11.1 fold, respectively, $p < 0.01$ vs. sham), when hyperalgesia and tactile allodynia were seen. However the MCH and 11CBY mRNA levels in the left (ligated) DRG return to the sham levels by day 14 after surgery. At all times after ligation, a decrease in the spinal cord MCH mRNA levels was observed, with a statistically maximal decrease (12.5 fold, $p < 0.01$ vs. sham) 28 days after surgery when compared to sham values. A statistically significant increase in MCH mRNA levels (4.45 fold, $p < 0.01$ vs. sham) 28 days after surgery in the right (non-ligated) DRG. This increase may represent a compensatory reaction to the decreased spinal cord MCH mRNA levels. Alternatively there are

several studies demonstrating cellular and molecular changes in the contralateral DRG following nerve injury (Oaklander, A.L. and Belzberg, A.J., Mol. Brain Res.(1997) 52: 162-165) and some of these changes might be related to the bilateral increase in trophic factor such as NGF after nerve injury (Koltzenburg M et al. TiNS (1999) 22:122-127). Also noted was an apparent decrease in the relative expression of MCH mRNA levels 24 hours timepoint in the right (non ligated) DRG. This most likely reflects lower quality RNA in this sample.

These data. indicate that MCH and 11CBy mRNA levels are modulated after peripheral nerve injury. In particular MCH may be involved in the long-term events that follow the partial nerve ligation.

SEQUENCE INFORMATION

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Claims

1. The use of a compound selected from:
   (a) an 11CBY polypeptide;
   (b) a compound which activates an 11CBY polypeptide; or
   (c) a compound which inhibits an 11CBY polypeptide; or
   (d) a polynucleotide encoding an 11CBY polypeptide,

for the manufacture of a medicament for treating:
   (i) stroke;
   (ii) pain; or
   (iii) neuropathies.

2. The use according to claim 1 wherein the medicament is used in the treatment of stroke.

3. The use according to claim 1 wherein the medicament is used in the treatment of pain.

4. The use according to claim 1 wherein the medicament is used in the treatment of neuropathies.

5. The use according to any one of claims 1 to 4 wherein the medicament comprises an isolated polypeptide which comprises a polypeptide having at least 95% identity to the 11CBY polypeptide of SEQ ID NO:2.

6. The use according to claim 5 wherein the isolated polypeptide is the 11CBY polypeptide of SEQ ID NO:2.

7. The use according to any one of claims 1 to 4 wherein the medicament comprises a compound which inhibits the activity of an 11CBY polypeptide

8. The use according to any one of claims 1 to 4 wherein the medicament comprises a compound which stimulates the activity of an 11CBY polypeptide

9. The use according to any one of claims 1 to 4 wherein the medicament comprises a polynucleotide encoding a polypeptide having at least 95% identity with the amino acid sequence of SEQ ID NO:2.

10. The use according to claim 9 wherein the polynucleotide comprises a polynucleotide having at least 95% identity with the polynucleotide of SEQ ID NO:1.

11. The use according to claims 9 or 10 wherein the polynucleotide has the polynucleotide sequence of SEQ ID NO:1.

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